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RNA Interference Therapeutics in Organ Transplantation

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Abbreviations

ALT, alanine aminotransferase

DC, dendritic cell

DGF, delayed graft function

dsRNA, double-stranded RNA

EVLP, *ex vivo* lung perfusion

HMGB-1, high-mobility group box 1

IRI, ischemia-reperfusion injury

LNP, lipid nanoparticles

MD-2, myeloid differentiation protein-2

MHC, major histocompatibility complex

mRNA, messenger RNA

mTOR, mammalian target of rapamycin

MyD88, myeloid differentiation primary response 88

NALP-3, NACHT domain, leucine-rich repeat domain, and pyrin domain-containing protein-3

PHD1, profyl hydroxylase domain enzyme 1

RISC, RNA-induced silencing complex

RNAi, RNA interference

SCS, static cold storage

shRNA, short hairpin RNA

siRNA, small interfering RNA

TLR, toll-like receptor

TRIF, TIR-domain-containing adapter-inducing interferon-β

TNFα, tumor necrosis factor alpha

Abstract

RNA interference (RNAi) is a natural process through which double-stranded RNA molecules can silence the gene carrying the same code as the particular RNA of interest. In 2006, the discovery of RNAi was awarded the Nobel Prize in Medicine and its success has accumulated since. Gene silencing through RNAi has been used successfully in a broad range of diseases, and, more recently, this technique has gained interest in the field of organ transplantation. Here, genes related to ischemia-reperfusion injury (IRI) or graft rejection may be silenced to improve organ quality after transplantation. Several strategies have been used to deliver siRNA, and pre-transplant machine perfusion presents a unique opportunity to deliver siRNA to the target organ during *ex situ* preservation. In this review, the potential of RNAi in the field of organ transplantation will be discussed. A brief overview on the discovery of RNAi, its mechanism and limitations are included. In addition, studies using RNAi to target genes related to IRI in liver, kidney, lung, and heart transplantation are discussed.

1. Introduction

Organ transplantation is often the only treatment for end-stage organ failure, such as for liver, kidney, lung, and heart. The ongoing discrepancy between transplant candidates and available organs has urged transplant centers to use more suboptimal organs, which are known to be more vulnerable to ischemia-reperfusion injury (IRI) compared to standard grafts. Mitigation of IRI should drive the utilization of suboptimal organs in order to address the shortfall in donor grafts. Hence, the evolutionary discovery of RNA interference (RNAi) holds potential to silence genes related to IRI and reduce graft injury after transplantation.[1] In addition, genes contributing to graft rejection may be silenced.

In this review, we will present a brief history of RNAi and describe the mechanisms by which gene silencing is induced. We will present an overview of recent literature on RNAi in the field of liver, kidney, lung, and heart transplantation, and we will focus on machine perfusion as a potential delivery platform for RNAi in organ transplantation. Finally, several limitations regarding clinical use of RNA interference are discussed.

1.1. RNA interference

In the early 1980s, it was revealed in *Escheria coli* that small RNA molecules can bind to a complementary sequence in messenger RNA (mRNA) and inhibit translation.[2] More than a decade later, the American scientists Fire and Mello were the first to describe that double-stranded RNA (dsRNA) molecules can silence the gene carrying the same code as the particular RNA of interest, the mechanism referred to as RNAi.[3] Despite the potency of RNAi, the technique could not yet be used for any therapeutic purposes in mammalian cells, because of long dsRNA activating a panic response in eukaryotic cells.[1] This problem was overcome with the discovery of so-called small interfering RNA (siRNA). It was demonstrated that after dsRNA enters the cell cytoplasm, an enzyme called 'Dicer' cleaves the dsRNA into smaller 21-23 base-pairs, called siRNA. Then, Argonaute protein in RNA-induced silencing complex (RISC) unwinds the double stranded siRNAs. One strand of the siRNA is removed (passenger strand), leaving the remaining strand available to bind to mRNA target sequences by Watson-Crick base pairing principle. The target mRNA is degraded, or its translation repressed, thereby blocking target gene's expression **(Figure 1)**.

In 2001, Elbashir and colleagues were the first to demonstrate that synthetic siRNA was able to induce RNAi in mammalian cells.[4] Since, a series of important and wide-ranging diseases have been targeted using siRNA-based therapies.[5] In 2006, the discovery of RNAi was awarded the Nobel Prize in Medicine, which further attracted a lot of research into this field. Another milestone was reached in 2018, when the United States Food and Drug Administration approved the first RNAi therapeutic treatment for a rare form of amyloidosis.[5] At least six other RNAi therapeutics are already in phase III clinical trials, of which one targets p53 to reduce the incidence of delayed graft function (DGF) after kidney transplantation. Nonetheless, the progress of siRNA has been a roller-coaster ride driven not only by science, but also regulatory trends, the stock markets, and big pharma businesses **(Figure 2)**.[6]

Currently, four types of small RNA are used in clinical trials, including siRNA, microRNA, short hairpin RNA (shRNA), and dicer substrate RNA.[5] These types differ in their biosynthetic pathway, the types of RISC they use, and the duration of silencing.

1.2. Delivery strategies for siRNA

The utility of RNAi therapy relies on effective delivery of the siRNA to the site of protein synthesis, and there are a number of factors that limit its therapeutic utility, such as unstable features of naked siRNA, extracellular and intracellular barriers, immune stimulation, and off-

target effects.[7] Chemical modification techniques can improve serum stability and increase the *in vivo* half-life and cellular uptake of siRNA. Other efficient ways to deliver siRNA molecules into host cells is by using viral vectors (e.g. lentiviral, adenovirus) or non-viral vectors (e.g. cationic lipids, polymers, and inorganic nanoparticles).[5] Viral vectors tend to have a higher transduction efficiency and a higher gene-silencing effect in most target cells compared to non-viral vectors. The downsides of viral vectors are however, the low specificity of the virus carrier, possible activation of the host cell oncogenes when a viral vector itself is randomly inserted in the host cell genome, and high costs. Multi-component non-viral vectors are encumbered by the need for intravenous administration, and in the case of lipid nanoparticles, pre-medication with steroids to mitigate infusion-related reactions. Also, exosomes are natural bio-carriers that have been investigated for the delivery of siRNA. Exosomes are suitable for hosting soluble drugs, have a high capacity for overcoming biologic barriers, cause few off-target effects, and have low immunogenicity. The therapeutic window of nanocarriers could be increased by targeted delivery, which can further reduce off-target effects and increase bioavailability of the therapeutic agent at the target site. Targeted delivery is usually achieved by adding a ligand, such as an aptamer or peptide, to the naked small RNA or to the nanoparticle.

In case of organ transplantation in particular, *ex situ* machine perfusion can be an attractive approach for targeted siRNA-delivery to the organ during preservation.[8] It prevents filtration by other organs, enzymatic degradation of siRNA in the serum, and off-target effects. Also, it is clinically more applicable, because RNAi-treatment to individual organs can take place after organ procurement, instead of treating the donor systemically. Thereby, ethical issues regarding premortem treatment of the controlled donation after circulatory are overcome, and regulatory issues in the multi-organ donor can be prevented so that only the target organ is treated by RNAi without the need for agreement of other transplant teams. After machine perfusion, the organ is flushed to remove the previously circulating machine perfusion solution, thereby also removing excess of circulating siRNA to prevent off-target effects in the recipient.

2. RNA interference and organ transplantation

In this section we will give an overview of the recent literature on RNAi in the field of solid organ transplantation. **Figure 3** shows the number of *in vivo* studies using RNAi in organ transplantation, and major events in the history of RNAi.

2.1. Liver transplantation

Several preclinical studies have been performed using RNAi to silence genes related to liver IRI **(Table 1).** Hence, Zhao et al. targeted high-mobility group box 1 (HMGB-1), an early mediator of post-reperfusion injury and inflammation, in a mice model.[9] The authors demonstrate improved liver function after reperfusion in the RNAi-treated group. Another study assessed the effects of siRNA targeting RelB, critical to both the regulation of apoptosis and increased expression of proinflammatory cytokines.[10] Systemic injection of RelB siRNA 24 hours before liver ischemia effectively reduced the level of RelB, and protected livers against IRI with reduced oxidative stress and a lower inflammatory response. Contreras and colleagues evaluated silencing of proapoptotic caspases 3 and 8 in a murine model of liver IRI.[11] The siRNA-treated mice showed a remarkable survival benefit with 30% of animals given caspase-8 siRNA and 50% of animals given caspase-3 siRNA surviving more than 30 days, whereas all of the control mice died within 5 days after the ischemic event.

Compared to siRNA, shRNA has also been used to target genes related to liver IRI. For example, silencing of NACHT domain, leucine-rich repeat domain, and pyrin domain-containing protein-3 (NALP3) was achieved in murine livers using shRNA against NALP3.[12] During IRI, NALP3 is pivotal in releasing inflammatory cytokines. Consequently, shRNA-treatment decreased release of cytokines, serum alanine aminotransferase levels (ALT), and inflammatory cell infiltration. Similarly, Hernandez-Alejandro et al. injected mice with tumor necrosis factor alpha (TNFα) shRNA 2 days prior to hepatic ischemia.[13] TNFα expression was significantly suppressed, and 6 hours after reperfusion, shRNA-treated mice showed lower peak ALT and improved liver histology compared to controls. Schneider and colleagues reduced expression of profyl hydroxylase domain enzyme 1 (PHD1) to protect hepatocytes against hypoxic damage and mitigate IRI.[14] Short hairpin RNA against PHD1 was administered intravenously with a viral vector in mice 5 days prior to ischemia. Serum ALT levels were reduced in shRNA-treated mice, and silencing of PHD1 attenuated hepatocyte death induced by IRI. While most small animal studies used hydrodynamic injection of siRNA, this approach is not practical for clinical use. High dosage can result in more off-target effects and large amounts of siRNA are costly. As such, lipid nanoparticles have attracted much interest given its high hepatic retention after intravenous administration.[15] In addition, Jiang et al. were the first to report on liver-specific liposome-based siRNA delivery to silence toll-like receptor (TLR) 4 in mice.[16] The authors modified the liposome

with galactose, which binds to the asialoglycoprotein receptor that is expressed on the surface of hepatocytes.

Recently, research from our group reported for the first time the use of siRNA during liver machine perfusion.[17] It was demonstrated that Fas siRNA directly added to the perfusion solution can be successfully delivered to rat liver grafts during both hypothermic and normothermic machine perfusion. Successful transfection into hepatocytes was achieved by coating siRNA with lipid nanoparticles (LNPs). Future studies using transplantation models should further investigate the effects of siRNA therapy during machine perfusion.

2.2. Kidney transplantation

The kidney could be an excellent target for RNAi therapy due to its unique characteristics of the urological system, which can lead to rapid uptake of siRNA. Various preclinical studies in kidney have been performed **(Table 2)** and, recently, a phase III multi-center trial to evaluate the inhibition of DGF by p53 siRNA in kidney transplant patients has been initiated by Quark Pharmaceuticals (www.clinicaltrials.gov).

Yang and colleagues have performed several studies aimed at silencing caspase 3, a key player in kidney IRI.[18–20] First, they showed renoprotection of naked caspase 3 siRNA in a porcine *ex situ* isolated reperfusion model.[20] Caspase 3 siRNA was infused into the renal artery before static cold storage (SCS) and added to autologous blood during 3-hour reperfusion. In the siRNA-treated group, apoptosis was significantly decreased, renal oxygenation was improved, and these kidneys showed a more favorable blood pH. In a follow-up study, using a porcine auto-transplantation model, they found that levels of caspase 3 were only downregulated during cold storage, but no longer after kidney transplantation.[18] The authors suggest poor stability of naked siRNA and/or systemic complementary effects overcoming the local effect. Concordantly, they performed a study using chemically modified naked caspase 3 siRNA in a porcine auto-transplantation model.[19] Caspase 3 siRNA was infused into the renal artery before SCS and administered intravenously to the recipient pig. As a result, caspase 3 was downregulated after SCS preservation and after transplantation, with improved renal function compared to controls.

Kidney IRI has also been associated with activation of the complement system. In a mice model, silencing of complement factor C5a was achieved by administering C5a siRNA two days before induction of renal ischemia.[21] Treatment with siRNA preserved renal function from IRI, as evidenced by reduced neutrophil influx and cell necrosis in renal tissue. Zheng et al. demonstrate

decreased complement-mediated IRI effects and a survival benefit after silencing complement 3 in mice.[22] The same group also used a combination of siRNAs targeting complement 3 and caspase 3, highlighting the potential of targeting multiple genes.[23] As such, Zhang et al. show a survival benefit after targeting both caspase 3 and caspase 8 in a mice clamping model.[24]

On another note, there is growing evidence that T cells participate in kidney IRI in an antigen-dependent manner. CD40 is a co-stimulatory molecule which participates in T-cell proliferation and other effector functions. Consequently, blockade of CD40 with siRNA improved renal inflammatory status in a rodent transplantation model.[25]

RelB and inhibitory kB kinase, both components of the NF-kB family, have also effectively been silenced in kidney models.[26,27] Survival experiments after RelB-siRNA show a huge advantage in 8-day survival rates: 80% in siRNA-treated mice compared to 10% of control mice.

Zheng et al. were the first to demonstrate gene silencing in a mice kidney transplantation model after kidney machine perfusion with a siRNA cocktail.[28] Treatment with siRNA targeting complement 3, RelB, and Fas decreased IRI, and improved kidney function and graft survival after transplantation. When cold ischemia time was prolonged, the siRNA-treated donor kidneys had much lower blood urea nitrogen and serum creatinine compared to controls, indicating that the siRNA cocktail attenuated renal dysfunction induced by prolonged cold ischemia time.

2.3. Lung transplantation

To achieve pulmonary delivery of siRNA, the most non-invasive way is through inhalation. Intratracheal administration and intranasal delivery are commonly used in animal experiments **(Table 3)**, but the clinical application of these routes is limited. Intratracheal administration uses an invasive setup and uncomfortable technique, whereas intranasal administration is disadvantageous in humans because our nasal cavity filters out the majority of particles. Therefore, pulmonary RNAi delivery systems should consider careful control of particle or droplet size to bypass the lungs natural defense mechanisms.

The Fas cascade is one of the most powerful pathways initiating apoptosis and inflammation in lung IRI. Del Sorbo et al. administered siRNA targeting Fas intratracheally in mice 2 days before inducing ischemia.[29] After reperfusion, silencing of Fas was found to decrease edema formation, improve lung compliance, and reduced cell apoptosis. Caspases also play a major role in the apoptotic cascade, and downregulation of caspase 3 was achieved by Zhang et al.

after administering shRNA in a rat model of lung IRI.[30] Animals treated with shRNA showed increased partial pressures of oxygen, and decreased partial pressures of carbon dioxide. In a rat autotransplantation model, Chi and colleagues used RNAi to target myeloid differentiation protein-2 (MD-2), which plays a key regulatory role in TLR activation and the inflammatory response during lung IRI.[31] Downregulation of MD-2 reduced water content of the lungs, and increased partial oxygen pressures with less acute lung injury after transplantation. Lv et al. used a model of rat lung transplantation to show successful silencing of p38 mitogen-activated protein kinase, which is a crucial signaling enzyme involved in inflammation and apoptosis.[32] Rats were administered p38a shRNA 2 days before ischemia, and treated animals showed a significant reduction in lung expression of p38a with reduced apoptosis and improved lung function.

Recently, the group from Hannover performed the first study using normothermic *ex vivo* lung perfusion (EVLP) to achieve so-called 'immunological invisibility' of the donor organ.[33] Silencing major histocompatibility complex (MHC) expression in lungs could lead to reduced graft rejection, thereby also reducing the hindersome side effects of lifelong immunosuppression. The study from Figueiredo et al. used delivery by lentiviral vectors with shRNA against swine leukocyte antigen during 2 hours EVLP. Their results demonstrated the feasibility of genetically engineering all lung regions. Moreover, perfusion with lentiviral vectors did not induce additional tissue injury, therefore not compromising the beneficial effect of EVLP.

2.4. Heart transplantation

Multiple studies have targeted genes related to ischemic injury after myocardial infarction, but only few reports focus on IRI in heart transplantation in particular **(Table 4)**. The unique structure of the cardiac muscle having a compact extracellular matrix filled with negatively charged molecules disrupts small RNA delivery using conventional carriers. Therefore, successful cardiac applications of RNAi must be preceded by the development of optimally designed delivery systems. A commonly used delivery method is by using facial amphipathic bile acids (deoxycholic acid) conjugated with a polymer.[34] Immune tolerance leading to permanent acceptance of heart transplants without immune rejection is a lofty goal for cardiac transplantation. To decrease graft rejection after transplantation, studies have targeted different TLRs and T-cell pathways using RNAi. In a mice model of heart transplantation, shRNA was used to block the co-stimulatory pathway B7/CD28/CTLA-4 through gene modification of dendritic cells (DCs).[35] Rats were infused by shRNA-transfected DCs, and successful immune tolerance of the graft was achieved.

Gene manipulation in DCs can decrease its allostimulatory capacity, which makes these cells more 'tolerogenic'. Besides, DCs express high levels of MHC molecules and costimulatory molecules CD40 and CD80, necessary for T cell proliferation, differentiation, and survival. Activation of T cells without co-stimulation may lead the development of immune tolerance. A study investigated silencing of both CD40 and CD80 by administering siRNA to the recipient 3 days prior to heart transplantation and up to 3 weeks after transplantation of a fully MHC-mismatched heart.[36] RNAi treatment significantly improved graft survival and 67% of recipients achieved tolerance to the transplanted heart. Zhang and colleagues blocked TLR-signaling by RNAi therapy against adaptor molecules myeloid differentiation primary response 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-β (TRIF).[37] Recipient mice were treated with MyD88 and TRIF siRNA vectors before and after transplantation of a fully MHC-mismatched heart. While untreated grafts only survived 5–8 days, siRNA treatment significantly prolonged graft survival with a median of 37 days. Several years later, Wang et al. also used RNAi to silence MyD88 and TIRF in a mice heart transplantation model.[38] In addition, they targeted mammalian target of rapamycin (mTOR), a critical signaling kinase. Graft survival was significantly extended compared to controls, and immune rejection was prevented through silencing of TRL and mTOR pathways.

Instead of treating the recipient, Zheng et al. preserved mice heart grafts in cold University of Wisconsin solution containing TNFα, complement 3, and Fas siRNAs.[39] Preservation in the siRNA-containing solution knocked down gene expression after transplantation. Moreover, treated heart grafts retained strong beating for more than 100 days, whereas all control grafts lost function within 8 days.

Recently, Wei et al., for the first time, used 3-hour hypothermic machine perfusion of mini pig donor hearts with a solution containing siRNAs targeting complement 3, caspase 8, caspase 3, and NF-kB-p65 genes.[40] Both *ex situ* reperfusion and orthotopic transplantation models showed beneficial effects after siRNA perfusion. RNAi therapy reduced cardiac IRI and improved graft function after transplantation.

3. Limitations of RNA interference therapeutics

Despite very promising therapeutic outcomes, widespread clinical applicability of RNAi is hindered by several limitations.[41] Stable delivery of small RNAs to the target cells/organ may be limited by filtering by the lungs, liver, or kidneys. Moreover, extracellular unstable small RNAs are highly susceptible to degradation by enzymes found in serum, which decreases *in vivo* half-

life. Small interfering RNA also has poor cellular uptake because of their high molecular weight and negative charge, so that they are not easily able to cross cell membranes via passive diffusion. Some RNAi-delivery strategies take advantage of endocytosis, but degradation or entrapment inside the endosome should be taken into consideration. Once in the cell cytoplasm, small RNAs remain vulnerable to degradation by intracellular RNAses and need to be recognized by and incorporated into RISC. It should also be noted that several ways of delivery that have been used in animal models (e.g. intratracheal delivery or intramyocardial injection) may not be applicable for clinical use.

Besides delivery challenges, RNAi might cause problems by silencing of non-targeted genes.[42] Off-target silencing is undesirable as it can lead to dangerous mutations of gene expression and unexpected cell transformation. Some small RNAs and miRNAs share their mechanisms of action. For example, long-term overexpression of shRNA may affect endogenous miRNAs, impeding their regulatory functions and inducing toxicity. This was supported by the observation that miRNAs in hepatocytes were downregulated after transfection with high levels of shRNA, leading to morbidity in mice.[43] However, the risk of oversaturating small RNA pathways can be minimized by optimizing dosage and sequence. Hence, monitoring and controlling intracellular siRNA levels is imperative for stable gene silencing, while mitigating adverse effects.

Though generally well-tolerated, small RNAs can activate the host immune response in a dose-dependent manner. Activation of the immune system might originate from the delivery vehicle (e.g. viral vectors) or from the process of RNAi itself. Studies have shown that siRNAs may trigger immune activation through interferon responses and TLR pathways.[44,45]

4. Conclusions

Since the discovery of RNAi, studies using this technique have rapidly accumulated in all fields of medicine. More recently, attention is given to RNAi as a technique to target genes related to IRI or graft rejection after organ transplantation and improve outcomes. It is exciting that first attempts have been made to translate RNAi therapy to the clinic, such as to decrease the incidence of DGF after kidney transplantation by silencing the p53 gene. However, a number of factors stand in the way of widespread therapeutic potential, such as stable delivery to the target cells/organ, immune responses, and undesired off-target effects. Hence, *ex situ* machine perfusion can offer a unique opportunity to deliver siRNA to the target organ with multiple benefits, such as prevention of systemic donor treatment, higher costs, and reduced off-target effects. Although the

results are promising, studies using RNAi during machine perfusion preservation are still very limited, and there are yet many uncertainties, such as optimal small RNA dosage, perfusion temperature, or perfusion duration. *Ex situ* organ machine perfusion is currently performed in kidney, lung, liver and heart transplantation and should be considered a realistic option for RNAi treatment since the benefits may be multiform. RNAi as a therapeutic agent is still in its infancy, but will definitely have an impactful effect in the field of organ transplantation in the near future.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation.*

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Figure legends

Figure 8.1. Schematic illustration of RNAi mechanism. Double stranded RNA (dsRNA) molecule binds to a Dicer protein, which cleaves it into small interfering RNA (siRNA). These siRNAs bind to an Argonaute protein, which is part of the RNA-induced silencing complex (RISC). The RISC separates the siRNAs in two strands: the passenger strand (blue) is degraded, while the guide strand (orange) serves as a search probe, which links RISC to complementary RNA targets. After this recognition target's expression can be regulated through several different mechanisms.

Figure 8.2. Major events on the histology of RNAi and the effect on market size, optimism, and visibility. *Figure adapted from Khvorova et al. [6].*

Figure 8.3. Major events in the history of RNAi. Events since the first description of RNAi in 1998 and the number of publications of *in vivo* studies RNAi in liver, kidney, lung, and heart transplantation.

Supporting information

Additional Supporting information may be found online in the supporting information tab for this article.

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Table 1. *In vivo* studies on RNA interference in liver transplantation

Model	Administration	Target	Type	Delivery method	Dose	Main outcome in treated groups compared to controls
Mice segmental hepatic IRI [9]	Intravenous injection 48 h before ischemia	HMGB-1	siRNA	PEI vector	50 μg	↓pathological damage, ↓expression of proinflammatory receptors
Mice segmental hepatic IRI [10]	Intravenous injection 24 h before ischemia	RelB	miRNA	Expression vector	50 μg	↓AST, ↓MDA, ↓MPO serum levels
Mice segmental hepatic IRI [11]	Intravenous injection 60 min before ischemia	Caspase 3, caspase 8	siRNA	Lipiodol	0.5 nmol	>30 days survival: 30% in caspase-8 treated, and 50% in caspase-3 treated. 0% survival on postop day 5 in controls.
Mice segmental hepatic IRI [12]	Intravenous injection 48 h before ischemia	NALP3	shRNA	Expression vector	100 μg	↓AST, ↓proinflammatory cytokines, ↓ inflammatory cell infiltration
Mice segmental hepatic IRI [13]	Intravenous injection 48 h before ischemia	TNFα	shRNA	Expression vector	50 μg	↓AST/ALT, improved histology, ↓MDA
Mice segmental hepatic IRI [14]	Intravenous injection before ischemia	PHD1	shRNA	Adenoviral vector		↓ALT, ↓oxidative stress, improved histology
Mice segmental hepatic IRI [16]	Intravenous injection 6 h before ischemia	TLR4	siRNA	Galactose-conjugated liposome nanoparticles	50 μg	↓ALT/AST, improved histology, ↓MPO, ↓MDA, ↓ROS, ↓inflammatory cytokines
Rat liver machine perfusion [17]	Hypothermic and normothermic machine perfusion	Fas	siRNA	Lipid nanoparticles	50 nM	Uptake of siRNA-lipid complexes by the liver
Rat liver transplantation [1*]	Portal vein injection 72 h before ischemia	LXRα		Lentiviral vector		↓fatty acid accumulation, improved survival
Rat segmental hepatic IRI [2*]	Intravenous injection 1 h before ischemia	HSP72	siRNA	Ultrasound microbubble contrast agent	20 μl	↓ALT, ↓TNFα. Ultrasound radiation improves gene transfection over micro-

						bubble only.
Rat liver transplantation [3*]	4-minute portal vein perfusion	IRAK-4	shRNA	Expression vector		Improved liver function and less histologic liver injury

ALT = alanine aminotransferase; AST = aspartate aminotransferase; HMGB-1 = high-mobility group box 1; HSP72 = heat shock protein 72; IRAK-4 = interleukin-1 receptor-associated kinase-4; IRI = ischemia-reperfusion injury; LXRα = liver X receptor alpha; MDA = malondialdehyde; MPO = myeloperoxidase; NALP3 = NACHT domain, leucine-rich repeat domain, and pyrin domain-containing protein-3; PEI = polyethyleneimine; PHD1 = profyl hydroxylase domain enzyme 1; ROS = reactive oxygen species; siRNA = small interfering RNA; shRNA = short hairpin RNA; TLR4 = toll-like receptor 4; TNFα = tumor necrosis factor alpha. References with * are stated in supplementary file 1.

Table 2. *In vivo* studies on RNA interference in kidney transplantation

Model	Administration	Target	Type	Delivery method	Dose	Main outcome in treated groups compared to controls
Pig kidney autotransplantation [18]	Infusion with UW into the renal artery during 24-hour SCS	Caspase 3	siRNA	Chemically modified (locked nucleic acid)	0.3 mg	Visual siRNA after SCS, but weak after transplantation
Pig kidney autotransplantation [19]	Infusion with UW into the renal artery during 24-hour SCS + intravenous injection in the recipient before transplantation	Caspase 3	siRNA	Chemically modified (locked nucleic acid)	0.3 mg	Caspase-3 knockdown after SCS and Tx. ↓apoptosis, ↓inflammation, improved renal function
Pig renal clamping and ex vivo reperfusion [20]	Infusion with UW into the renal artery during 24-hour SCS. Infusion into autologous blood.	Caspase 3	siRNA	Chemically modified (locked nucleic acid)	3 μg/ml, 0.15 μg/ml	↓apoptosis, ↓inflammation, doubled in oxygen consumption, neutralized perfusate pH, ↑renal blood flow
Mice renal clamping [21]	Intravenous injection 48 h before ischemia	C5a	siRNA	Expression vector	50 μg	↓BUN, ↓serum creatinine, ↓proinflammatory cytokines, ↓neutrophil influx, ↓cell necrosis
Mice renal clamping [22]	Intravenous injection 48 h before ischemia	C3	shRNA	Expression vector	50 μg	↓complement-mediated IRI, improved renal function, ↓mortality
Mice renal clamping [23]	Intravenous injection 48 h before ischemia	C3 and caspase 3	siRNA	Expression vector	50 μg	↓BUN, ↓creatinine, ↓mortality. Reduction in histopathological injury.
Mice renal clamping [24]	Intravenous injection 48 h before ischemia	Caspase 3 and 8	siRNA	Expression vector	50 μg	Significant survival benefit
Rat kidney transplantation [25]	Intravenous injection 1 h before nephrectomy in the donor and before reperfusion in the recipient	CD40	siRNA	Chemically stabilized (backbone modification)	15–500μg	Improved renal inflammatory status, ↓TNFα. Renoprotection after transplantation
Rat renal clamping [26]	Renal artery injection 48 h before	IKKβ	siRNA	Naked	0.25 mg/kg	↓BUN, ↓serum creatinine, ↓renal tubular

	ischemia					damage scores
Mice renal clamping [27]	Intravenous injection 48 h before ischemia	RelB	siRNA	Expression vector	50 μg	90% of control mice died from lethal IRI, 80% treated mice survived up to day 8
Mice kidney transplantation [28]	Hypothermic kidney perfusion for 4 hours	C3, RelB, Fas	siRNA	Naked	100 μg	Controls died around day 40, compared to treated animals who survived >80 days
Rat kidney transplantation [*4]	*In situ* perfusion with UW solution	SHARP-2	shRNA	Lentiviral construct		Prolonged survival by 4–5 days
Mice renal clamping [*5]	Transarterial injection after IRI	p53	shRNA	Expression vector, polymer	0.33 μg / 3.3 μg	↓apoptosis, ↓creatinine, improved histology
Rat renal clamping [*6]	Intravenous injection between 16 h before – 8 h after ischemia	p53	siRNA	Lipid nanoparticle	12 mg/kg	Rapid delivery of siRNA to proximal tubule cells, ↓apoptosis, improved histology
Mice renal clamping [*7]	Inferior vena cava delivery 48 h before ischemia	Fas and caspase 8	shRNA	Expression vector	150 μg	↓BUN, ↓creatinine, ↓renal tubular injury
Mice renal clamping [*8]	Intravenous injection 48 h before ischemia	TNFα	siRNA	Expression vector	50 μg	90% of control mice died from lethal IRI, 50% treated mice survived up to day 8

BUN = blood urea nitrogen; C3 = complement 3; C5 = complement 5; SCS = static cold storage; siRNA = small interfering RNA; SHARP-2 = split- and hairy-related protein 2; shRNA = short hairpin RNA; TNFα = tumor necrosis factor alpha; UW = University of Wisconsin; IKK = inhibitory kB kinase; IRI = ischemia-reperfusion injury. References with * are stated in supplementary file 1.

Table 3. *In vivo* studies on RNA interference in lung transplantation

Model	Administration	Target	Type	Delivery method	Dose	Main outcome in treated groups compared to controls
Mice lung ischemia and *ex situ* reperfusion [29]	Intratracheal treatment 48 h before ischemia	Fas	siRNA	Naked	100 µL	↓edema, improved lung compliance, ↓apoptosis
Rat lung clamping [30]	Intratracheal treatment 48 h before ischemia	Caspase 3	shRNA	Viral vector	30 µg	Protection lung function, ↑ paO_2, ↓ $paCO_2$
Mice lung transplantation [31]	Endotracheal treatment 48 h before ischemia	MD-2	siRNA	Lipid vector		↓pathology of acute lung injury, improved paO_2, ↓proinflammatory cytokines
Rat lung transplantation [32]	Intratracheal treatment 48 h before ischemia	p38	shRNA	Expression vector	2 µg	↑pulmonary vein O_2 level, ↓wet weight-to-dry weight ratio, ↓lung injury score, ↓proinflammatory cytokines, ↓apoptosis
Pig machine perfusion [33]	2-hour normothermic EVLP	SLA I and II	shRNA	Viral vector		Feasibility of genetical engineering of all lung regions. Silencing >50%. No effect on cell viability of tissue integrity.

MD-2 = myeloid differentiation protein-2; $paCO_2$ = partial carbon dioxide pressure; paO_2 = partial oxygen pressure; shRNA = short hairpin RNA; siRNA = small interfering RNA; SLA = swine leukocyte antigen

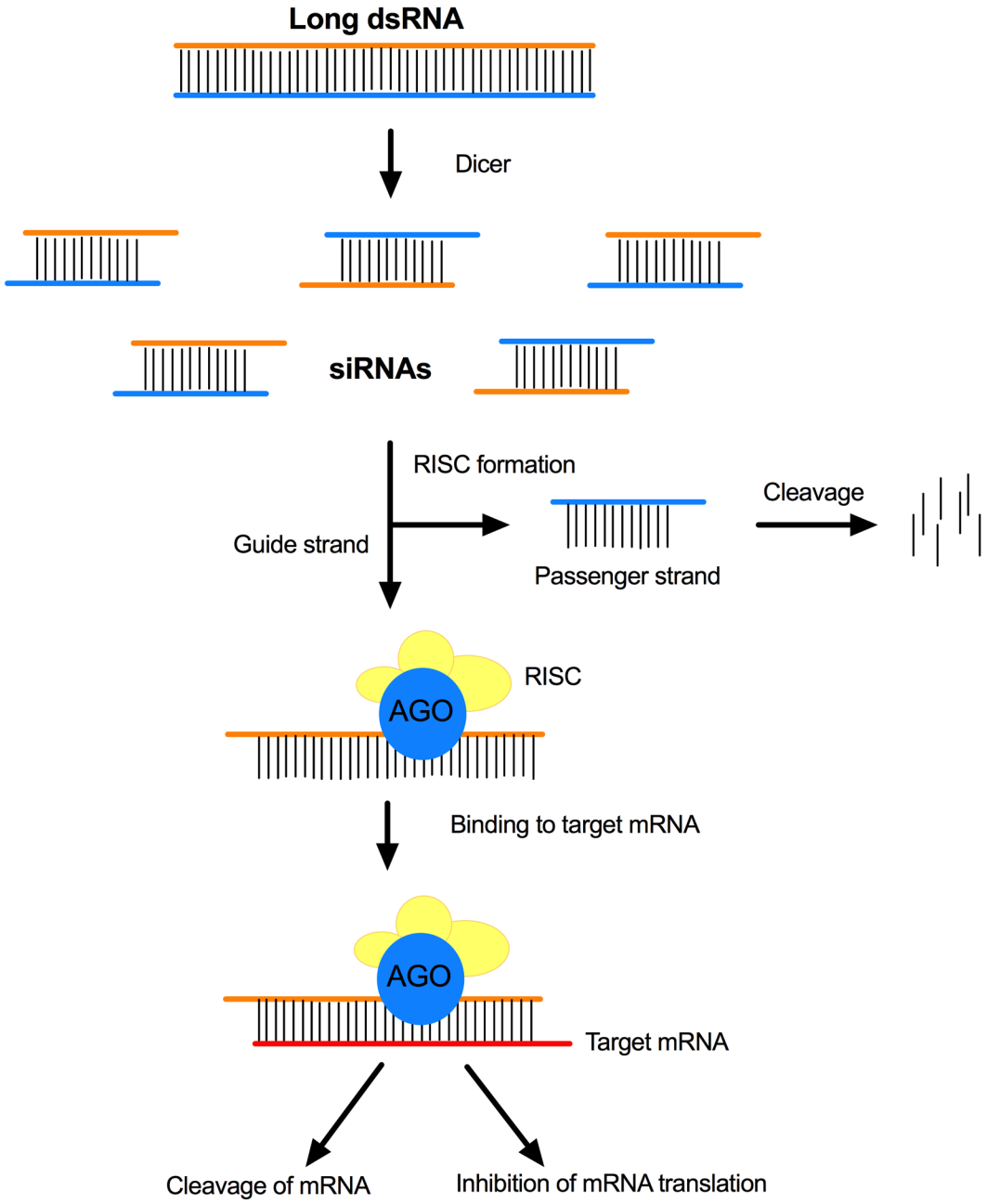
Table 4. *In vivo* studies on RNA interference in heart ischemia and transplantation

Model	Administration	Target	Type	Delivery method	Dose	Main outcome in treated groups compared to controls
Mouse heart transplantation [35]	Intramyocardial injection immediately after ischemia	B7/CD28	shRNA	Expression vector	100 μg	↑survival time, ↓organ rejection
Mice heart transplantation [36]	Intravenous injection 3 days before transplantation, and 7, 14, 21 days after transplantation	CD40/CD80	shRNA	Expression vector	50 μg	↑survival
Mouse heart transplantation [37]	Intravenous injection 3, 7 days before transplantation and 7, 14 days after transplantation	MyD88, TRIF	siRNA	Expression vector		Prolonged graft survival
Mouse heart transplantation [38]	Intravenous injection 3, 7 days before transplantation and 7, 14, 21 days after transplantation	MyD88, TRIF, mTOR	siRNA	Expression vector	50 μg	Prolonged graft survival
Mouse heart transplantation [39]	Preservation in UW solution	TNFα, C3, Fas	siRNA	Expression vector	2 μg	Strong heart beats >100 days after transplantation in treated animals versus loss of graft function <8 days in controls.
Pig ischemia and *ex vivo* reperfusion/ transplantation [40]	Cold Celsior preservation solution	C3, caspase 3 and 8, NF-kB-p65	siRNA	Expression vector		↓apoptosis, ↓myocardial damage, ↓tissue inflammation, improved cardiac function
Rat myocardial LAD ligation and reperfusion [*9]	Intramyocardial injections 4 days before ischemia	CD47	siRNA	Viral vector		↓infarct size, ↓serum myocardial enzymes, ↑activity of eNOS, ↑NO, ↓oxidative stress

Rat myocardial LAD ligation and reperfusion [*10]	Intramyocardial injection	RAGE	siRNA	PEI and membrane-penetrating delivery	150 μg	↓proinflammatory cytokines, ↓apoptosis, ↓myocardium fibrosis
Rat myocardial LAD ligation and reperfusion [*11]	Intramyocardial injections 1 h before ischemia	SHP-1	siRNA	DA-PEI vector	100 μL	↓apoptosis, ↓infarct size
Rat heart ischemia and *ex vivo* reperfusion [*12]	Intramyocardial injection after ischemia	CypD	siRNA	Viral vector		Dose-dependent protective on mitochondrial membrane potential
Mouse myocardial LAD ligation and reperfusion [*13]	Intramyocardial injection 10 min after ischemia	PHD2	shRNA	UTMD	25 μg	Improved fractional shortening, ↑presence of small capillaries and venules in the infarcted zones, ↑HIF-1
Rat myocardial LAD ligation and reperfusion [*14]	Intravenous injection 10 min after ischemia	PHD2	shRNA	UTMD	120 μL	↑HIF-1, ↓infarct size, improved heart function
Mouse heart transplantation [*15]	Intramyocardial injection 1 h after ischemia	PHD2	siRNA	Arg-G4 nanovector	10–200 nM	PHD silencing in MSCs, ↓apoptosis, ↓fibrosis, ↑angiogenesis, ↓ventricular remodeling, improved heart function

C3 = complement 3; CypD = cyclophilin D; DA = deoxycholic acid; eNOS = endothelial nitric oxide synthase; HIF-1 = hypoxia inducible factor 1; LAD = left anterior descending artery; PHD2 = profyl hydroxylase domain enzyme 2; MSC = mesenchymal stem cell; mTOR = mammalian target of rapamycin; MyD88 = myeloid differentiation primary response 88; NO = nitric oxide; PEI = polyethyleneimine; RAGE = receptor advances glycation end products; siRNA = short interfering RNA; SHP-1 = Src homology region 2 domain-containing tyrosine phosphatase;

shRNA = short hairpin RNA; TNFα = tumor necrosis factor alpha; TRIF = TIR-domain-containing adapter-inducing interferon-β; UTMD = ultrasound-targeted microbubble destruction; UW = University of Wisconsin. References with * are stated in supplementary file 1.



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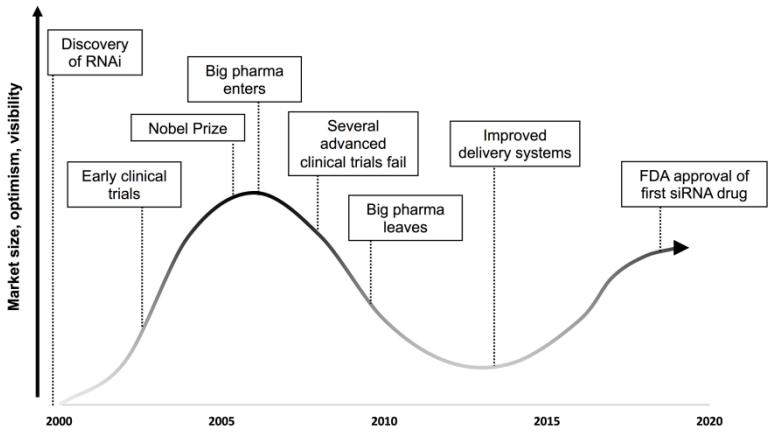

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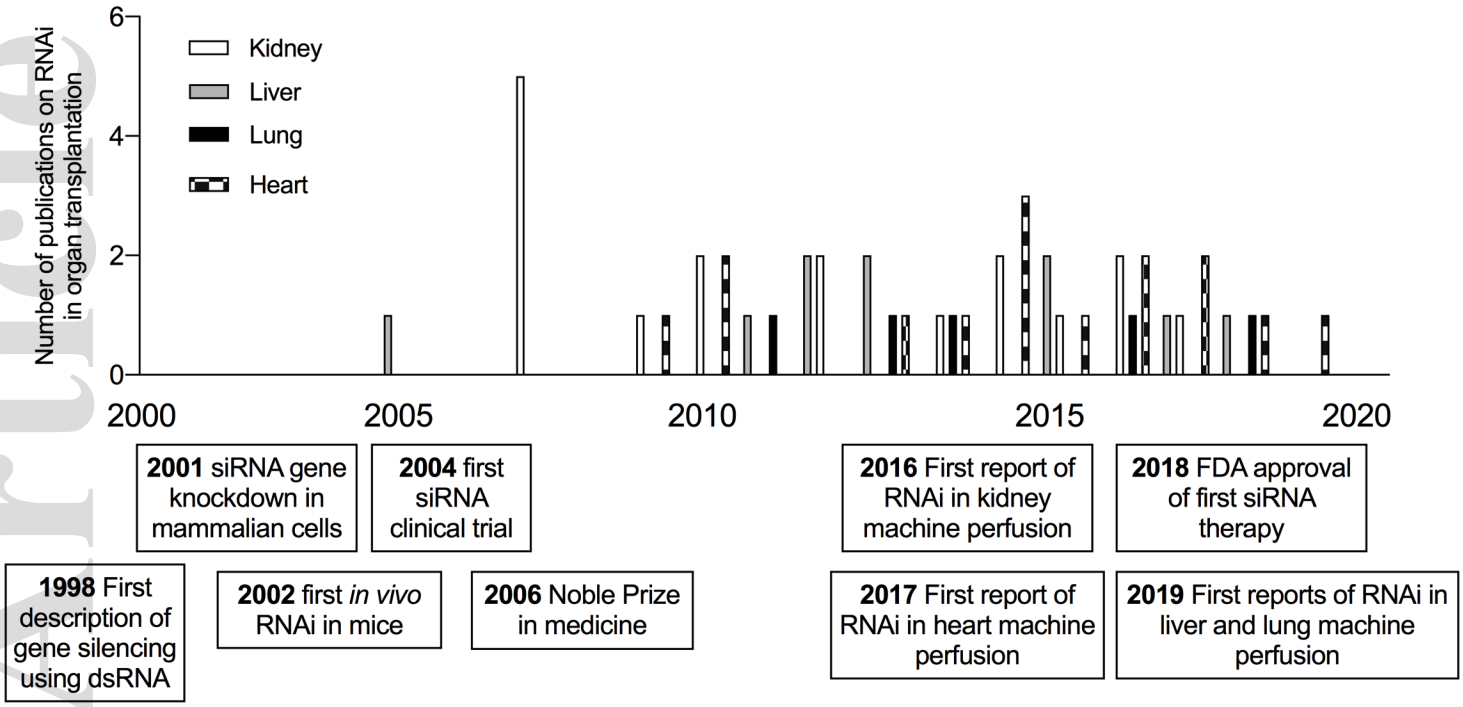

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